CLONING OF DNA METHYLTRANSFERASE 3A and 3B

FOR PARTIAL FULFILLMENT
OF THE MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

Submitted by
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2011-2012


Dedicated
to my
beloved
parents

DEPARTMENT OF LIFESCIENCE
NATIONAL INSTITUTE OF TECHNOLOGY
ROURKELA-769008

.....

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Associate Professor and Head. **Date: .....**

CERTIFICATE

This is to certify that the thesis entitled **"Cloning of DNA methyltransferase 3A and 3B in Human Cancer"** which is being submitted by **Miss. SobhaBiswal,** Roll No. **410LS2045,** for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafied research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I hereby declare that the thesis entitled "Cloning of DNA methyltransferase 3A and 3B", submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bona fied and original research work carried out by me under the guidance and supervision of Dr. Samir Kumar Patra, Associate Professor and Head of Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other institutes or organization for the award of M Sc. degree.

Date:~
Place:~

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Lastly, I acknowledge with highest sense of regards to my parents, my brother, sister and other members of my family for their supreme sacrifice, blessings, unwavering support, love and affection without which the parent investigation would not have been successful in any sphere of my life.

Date:~
Place:~

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ABBREVIATION

µ	Micro
:	Ratio
%	Percentage
µl	Microliter
µg	Microgram
bp	Base pare
cDNA	Complimentary DNA
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acitic acid
gm	Gram
LB	Luria-Bertani
Fig.	Figure
viz.	Such as
/	Per
V	Volt
Ng	Nanogram
PCR	Polymerase Chain Reaction
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycitidine 5'-triphosphate

dGTP	2’-deoxyguanosine 5’-triphosphate
DEPC	Diethyl Pyrocarbonate
GC	Guanine and Cytosine
OD	Optical density
UV	Ultra Violet
CFU	Colonies forming unit
mRNA	Messenger RNA
RNase	Ribonuclease
RT	Reverse Transcriptase
RPM	Revolution Per Minute
TBE	Tris Borate EDTA
U	Unit
NCBI	National Center for Biotechnology Information
IPTG	Isopropyl β-D thyogalactoside
X-gal	5-bromo,4-chloro,3-indolyl β-D Galactopyranoside
et.al	And others

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1. ABSTRACT

ABSTRACT

DNA Methylation acts as an essential regulatory mechanism of transcription affecting chromatin structure, which is established and properly maintained by the co-ordinate action of three DNA methyltransferases: DNMT1, DNMT 3A and 3B and histone modifications.DNMT1 is constitutively expressed and is required for the maintenance of global methylation after DNA replication. In contrast, the DNMT3 family genes appear to be developmentally regulated and behave like de novo DNA methyltransferases in vitro . Recent studies show that epigenetic change plays a major role in silencing a variety of methylated tissue-specific and imprints genes in many cancer types. The DNMT3A and DNMT3B show high homology in the carboxy terminal catalytic domain and contain a conserved cysteine rich region which allocate homology with the X-linked ATRX gene of the SNF2/SWI family. In this study, we have cloned human DNMT3Aand DNMT3B. This cloning of the human DNMT3 genes will facilitate further biochemical and genetic studies of their functions in establishment of DNA methylation patterns, regulation of gene expression and tumorigenesis.

Key words:- DNA Methylation, DNMTs, ATRX gene, SNF2/SWI family

2. INTRODUCTION

The term “epigenetic” was coined by Conrad Waddigton in 1940s according to him “Epigenetics is the study of heritable changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence”. These alterations may remain through out the cell divisions for the remainder of the cell's life and may also last for multiple generations . Epigenetic be noticeable in the literature as far as the mid 19th century, although the conceptual origins data back to Aristatol (384-322 B.C). He conceived in epigenesist in the development of individual organic form from the unformed. This argumental view was the main argument against our having developed from miniscule fully formed bodies. Epigenetics is appear to form and take shape as a new scientific discipline, which will have deep impact on Medicine and Approximately all fields of biology.

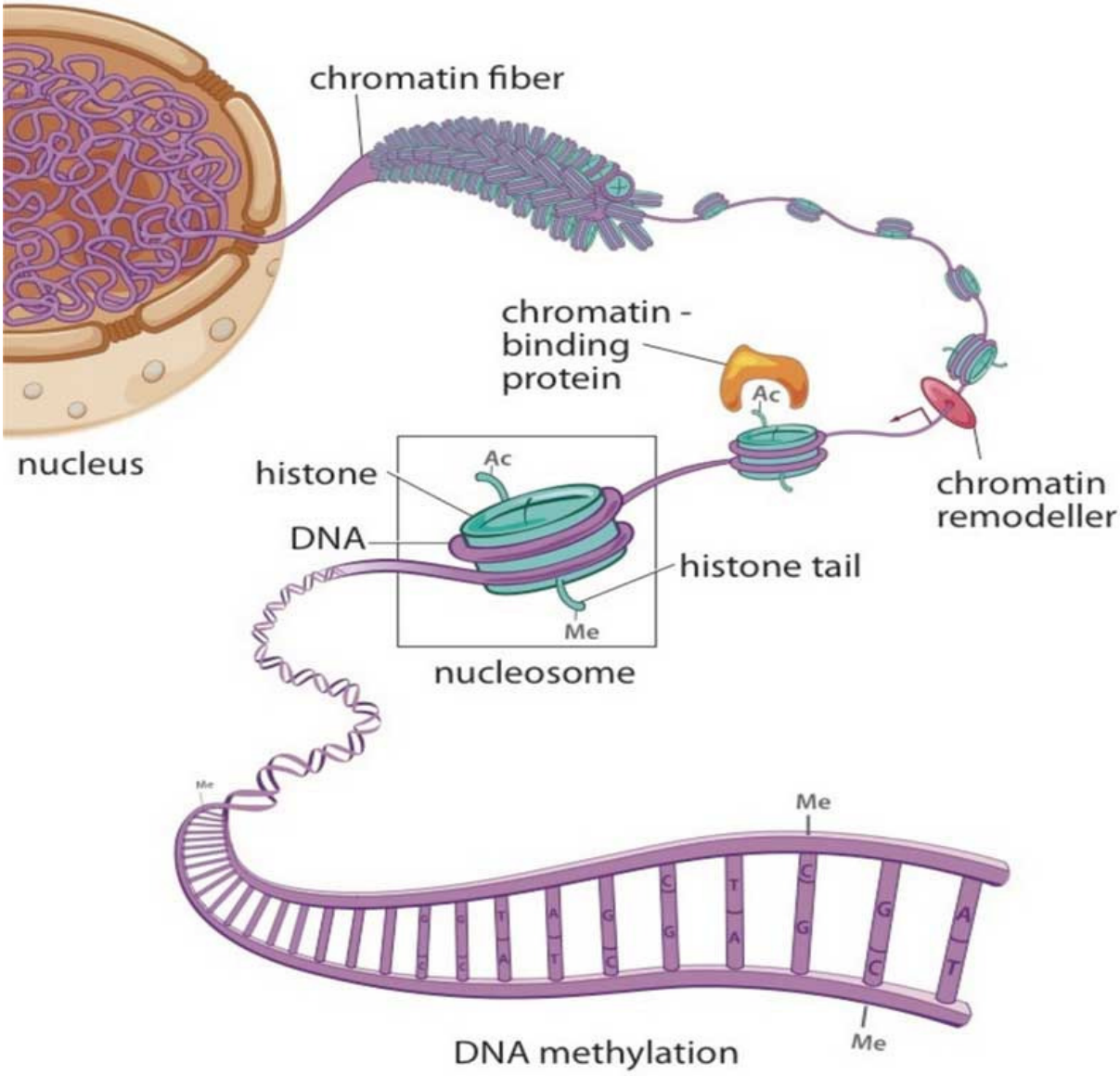

Figure1. Epigenetic modification (Madame Curie Bioscience and database)

Epigenetics covers many different levels of biological control from variegated expression in Drosophila to chromatin structure and modification down to DNA methylation and relates to different biological systems. Epigenetics affect the transcription in the cell, thereby managing gene expression and abnormal epigenetic changes can have serious effects for the organism. Most epigenetic modification only occur within the course of one individual organism's lifetime, but if a alteration in the DNA has been caused in gamete that result in fertilization, then some epigenetic modification are inherited from one generation to the next - "Lamarckism". We can very roughly branch epigenetics into three substantially overlapping categories: DNA methylation, genomic imprinting, and histone modification. Among these mechanisms, DNA methylation is the main focus of my thesis. The epigenome is comprised of chromatin and its associated proteins. Pattern of covalent modifications of DNA by methylation setup and maintains gene expression patterns.

3. REVIEW OF LITERATURE

Review of literature

In the post genomic era of cancer biology it is becoming increasingly evident that epigenetic controls of gene expression play an important role in determining the phenotype of cancer cell. Maintenance of balance between cell proliferation and cell death is the main key of appropriate development. Cancer cells have higher rate of proliferation than death.(Patra, S. K. Biochimica et Biophysica Acta 1785 (2008) 182–206). Histone modifications and DNAmethylationand demethylation events are central to the epigeneticregulations of development [Reik,W et al 2001, Surani et al 2002, Judso et al 2002, Hata et al 2002,Bourc'his,D. Et al 2001, Mc Lay et al 2003, Haaf, t. Et al 2006]. The mechanisms controlling these events and their dynamic changes have important implications in developmental cell biology as well as carcinogenesis and tumour progression.

DNA methylation

Methylation of DNA was discovered by R.D. Hotchkiss while separating and quantifying DNA bases from nuclear preparations.(Hotchkiss .R.D et al, 1948)DNA methylation acts an powered role in regulation of gene expression (Kass et al., 1997), genomic-imprinting (Bartolomei and Tilghman, 1997), and Xchromosome inactivation (Jaenisch et al., 1998), and has been shown to be main point for mammalian development (Li et al., 1992).Mammalian cells have the capacity to epigenetically modifytheir genomes via DNA methylation. Methylation at the C-5 position of cytosine within CG dinucleotidesis an epigenetic modification critical for normal development,differentiation, gene regulation and control of chromatinstructure in mammalian cells .

5 methylcytosines account for about 1% of total DNA bases in the human genome and affects 70-80% of the CpG sites in a human somatic cell (M Ehrlich at al 1986). Approximately 70% of the CpG residues in themammalian genome are methylated, however the allocation of CpG is not random and the majority of the genome is CpG-poor(Cooper et al 1989). Certain regions of the genome which are often, but not always, clustered at the 54-ends of genes possess the probable CpG frequency and have been termed CpG islands . CpG methylation has been collaborative with reduced transcription (Bird, A. P et al 1992, Kass, S. U et al 1997, Razin, A. Et al1998), decreased DNase I sensitivity (Keshet, I et al 1986), and decreased site-specific recombination (Hsieh, C-L at al 1992).DNA methylation is a covalent modification of nucleotides and the most frequently occur in cytosine bases of DNA sequence. The cytosine is methylated in the C-5 position byC-C covalent bonding with the

help of universal methyl donor Sadenosyl- L-methionine (SAM) in association of a family of DNA (cytosine-5) methyltransferases (DNMTs) and here SAM changes to S-adenosylhomocystein (Razin,A et al 1984, Adams, R.L.P et al1985,Jost et al1993,Jones et al 2007, Christman et al 2002, S.K Patra et al 2008).

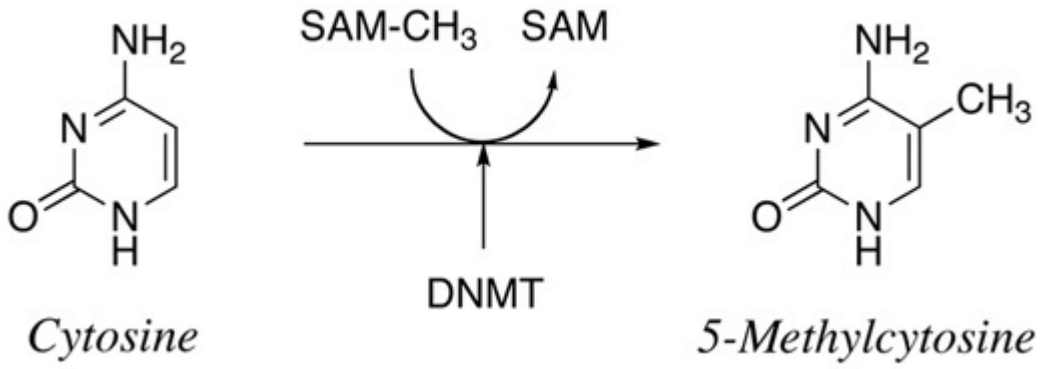

Figure.2: Methylation of cytosine catalyzed by DNMTs. (C.P. Walsh and G.L. Xu. Curr Top Microbiol Immunol., 2006, 301, 283-315)

DNA Methyltransferases (DNMTs):-

The mammalian DNMTs family encompasses DNMT1, DNMT2, DNMT 3. DNMT 3 family consists of two related genes, termed DNMT 3A and DNMT 3B.

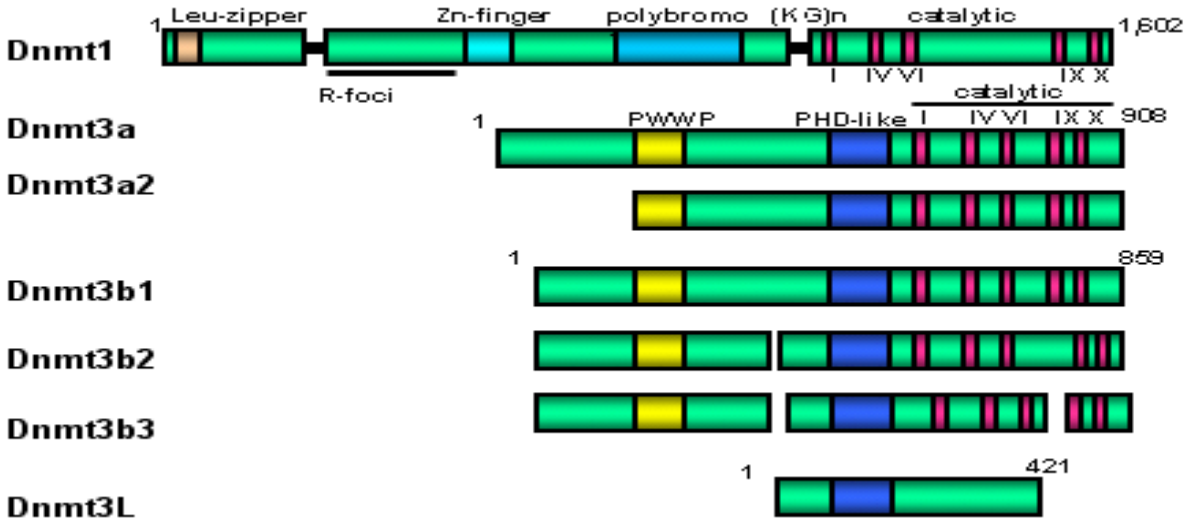

Figure.3: Members of mammalian DNMTs family(Takeshima et al. (2006) J. Biochem. 139, 505-515.)

This family is divided into maintenance and de novo Methyltransferases. DNMT1 is specialized to carry out most of the maintenance methylation following DNA replication, whereas DNMT3A and DNMT3B are capable for de novo methylation during embryogenesis and germ cell development (Cooper et al 1989) Maintenance DNMT1 binds methyl groups to the hemimethylated DNA in the time of replication, where as de novo DNMT3A and DNMT3B add methyl groups to CpG dinucleotides of unmethylated DNA .DNMT1, DNMT3A and DNMT3B are required for formation of the established pattern of methylation in promoters and first exons of human genomic DNA (Das PM et al 2004). Hypermethylation and repression of selective genes by ectopic expression of DNMTs be present unclear. Present molecular genetic and enzymatic/proteomic data suggest that DNMT1 and DNMT3a trigger class I histone deacetylases (HDACs) to precipitate inactive chromatin. (Patra S.K. et al 2001) Among these my topic based on DNMT 3A and DNMT 3B.

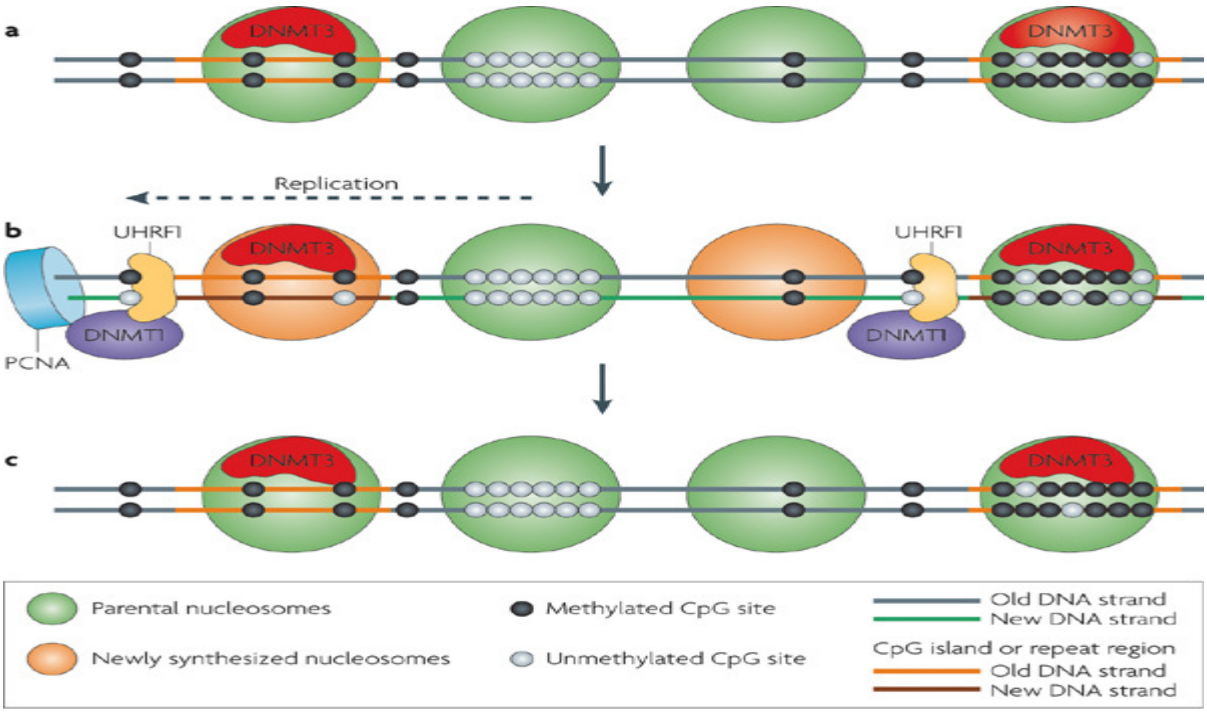

Fig.4:-Pattern of DNA Methylation (Peter A. Jones & Gangning Liang

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DNMT3A and DNMT3B is active without interaction with their N-regulatory regions. The N-terminal domain possesses nuclear localization signal sequence (NLS) responsible for localization of DNMTs in the nucleus. The N-terminal end of of DNMTs also contains proliferating cell nuclear antigen binding domain (PDB), a cysteine rich zinc finger DNA binding motif (ATRX), and polybromo homology domain (PHD) targeting DNMTs to the replication foci. However, PWWP tetra peptide is only present in N-terminal domains of DNMT3A and DNMT3B and interact with histones (Hermann A et al 2004). The C-terminal domain contains six motifs I, IV, VI, VIII, IX and X. Motifs I and X form S-adenosylomethionine binding site, motif IV associate with cytosine at the active site, motif VI possesses glutamyl residue giving protons, and motif IX maintains the structure of the target recognition domain (TRD) usually spotted between motifs VIII and IX, that form base-specific contacts in the chief groove of DNA (Bestor TH et al 2000, Robertson KD et al 1999, Weisenberger DJ 2004 , . Xie S et al 1999)

DNMT 3A

DNA (cytosine-5)-methyltransferase 3A is an enzyme that in humans is encoded by the DNMT3A gene. This gene encodes a DNA methyltransferase that do function in de novo methylation.The protein localizes to the cytoplasm and nucleus and its expression is developmentally balanced. Another splicing results in multiple transcript variants encoding different isoforms. It was identified by EST database searces in 1998 using the conserved methyltransferase motif as bait. It is found in human chromosome 2p23. It has 96% aminoacid identity with murine dnmt3a gene. DNMT 3A is ubiquitously expressed in adults tissues , most tumor types , cell embryos and embryonic stem cells.

Structure of DNMT 3A:-

It contain an Amino terminal domain and a carboxyterminal domain.

(a) Amino terminal domain

It contains a PWWP domain ant a ATRX motif in the N-terminal

PWWP domain: A domain contain a highly conserved “proline- tryptophan-tryptophan-proline” motif involved in heterochromatin association.

ATRX motif: An ATRX related cystein rich region containing a C2-C2 zinc finger and an atypical PHD (Plant homo domain) implicated in protein protein interaction.

This characterise that DNMT 3A and DNMT 3B may be associated with structural change in chromatin via protein-protein interactions at the amino terminal region.

(b) Carboxyterminal domain

The catalytic motifs are highly conserved in all cytosine DNMTs.

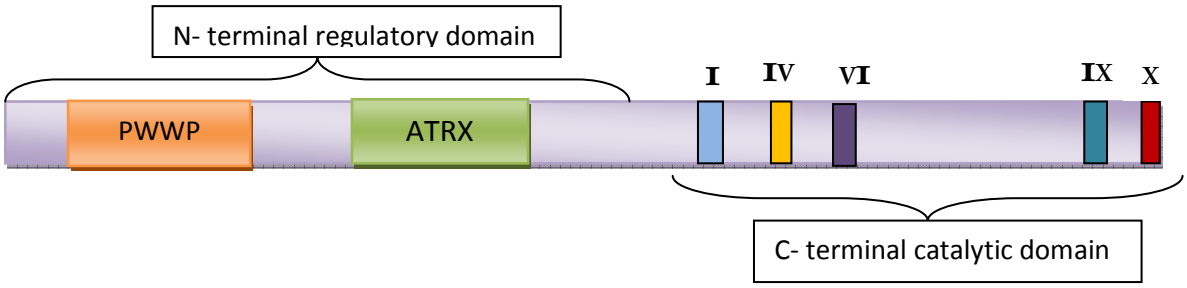


Figure.5**: Structure of DNMT 3A** (Atlas of Genetics and Cytogenetics in Oncology and Haematology)

Function:

Essential for genome wide de novo methylation and it is essential for the establishment of DNA methylation patterns during development process. Methylation of DNA is coordinated with methylation of histones. It changes DNA in a non-processive manner and also methylates non-CpG sites. It may mostly methylate DNA linker between 2 nucleosomal cores and is repressed by histone H1. Plays a crucial role in paternal and maternal imprinting. This is essential for methylation of most imprinted loci in germ cells. It acts as a transcriptional co-repressor for ZNF238.Can actively repress transcription through the recruitment of HDAC activity.DNA methyltransferase (DNMT) add methyl groups to DNA to effect gene expression. Basically there are three main types of DNMTs. DNMT1 is predominately capable for hemi-methylated CpG island methylation, DNMT2 transfers methyl groups to RNA in state of DNA. Hence, has been renamed to t-RNA aspartic acid methyltransferase 1 (TRDMT1) and DNMT3 is responsible for unmethylated CpG island methylation.

DNMT 3B

DNA (cytosine-5)-methyltransferase 3 beta, also known as DNMT3B, is a protein associated with immunodeficiency, centromericdivergence and facial anomalies syndrome. CpGs methylation is a type of epigenetic modification that is important for embryonic development,

imprinting, and X-chromosome inactivation. DNMT3B gene encodes a DNA methyltransferase which is thought to function in de novo methylation, rather than maintenance methylation. This protein localizes primarily to the nucleus and its expression is developmentally regulated. It was identified by EST database searches in 1988. This gene is mapped to human chromosome 20 q11.2 . It has 85% amino acid identify with DNMT 3B of mouse. DNMT is expressed high levels in testes and play a crucial role in spermatogenesis.

Structure of DNMT 3B:-

It contain an Amino terminal domain and a carboxyterminal domain.

(a) Amino terminal domain

It contains a PWWP domain and a ATRX motif in the N-terminal.

(b) Carboxyterminal domain

The catalytic motif are highly conserved in all cytosine DNMTs.

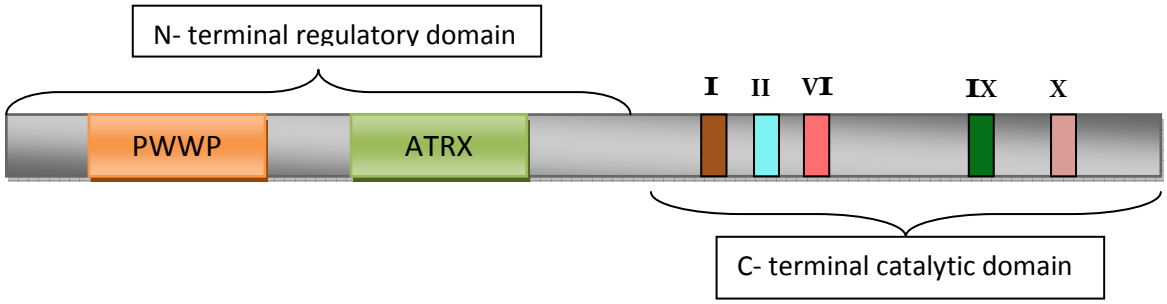


Figure.6 **: Structure of DNMT 3B** (Atlas of Genetics and Cytogenetics in Oncology and Haematology)

Function:

DNMT3B both maintain DNA methylation and gene silencing in human cancer cells. DNMT3B may not only silence genes by direct DNA methylation or recruitment of proteins that for chromatin modification, but also play an essential role in transformation .Required for genome wide de novo methylation and is essential for the establishment of DNA methylation patterns during development. DNA methylation is associated with methylation of histones. This preferentially methylates nucleosomal DNA within the nucleosomal core region. This May function as transcriptional co-repressor by associating with CBX4 and independently of DNA methylation involved in gene silencing. In association with DNMT1 & and via the recruitment of CTCFL/BORIS associate in activation of BAG1 gene expression by modulating demethylation of promoter histone H3 at H3K4 and H3K9. Isoforms 4 and 5 are hopefully not functional due to the

deletion of two conserved methyltransferase motifs. DNA-methyltransferase-3B carried out an important role in the generation of aberrant methylation in carcinogenesis. Polymorphisms and haplotypes of the DNMT3B gene may influence DNMT3B activity on DNA methylation, thereby altering the affectivity to lung cancer. The DNMT3B gene has two transcriptional start sites, which exist in different exons (exon 1Aand1B) and the expression is regulated by variety of promoters. One promoter is nested within a CpG-rich area, whereas the additional promoter is found in CpG poor. (Lee, *et al*., volume 26)

4. OBJECTIVES OF THE PROJECT

OBJECTIVE

DNA methylation and DNA demethylation are two different, exactly opposite, conceptually defined chemical reactions similar to other epigenetic modifications such as acetylation and deacetylation, phosphorylation and dephosphorylation, which are frequently encountered while studying the dynamics of the epigenome.

Keeping this hypothesis in mind and based on the literature survey, the present work has been undertaken on two proteins DNMT 3A and 3B for their further characterisation. And the objectives of the project include:-

Cloning of DNMT3A and DNMT3B.

5. Material and Methods

Material and Methods

RNA extraction:-

Extraction from Blood By RNA Purification Kit:-

The collected cancer blood was centrifuged at 3000 rpm for 15mins at 4° C. The supernatant containing the serum was separated from the pellet which contains the blood cells. The pellet was resuspended in 600µl of Lysis Buffer (supplemented with 20µl of 14.3 M β-mercaptoethanol for every 1ml of Lysis Buffer) and vortexes to mix thoroughly. 450µl of ethanol (96-100%) was mixed with the solution. About 700µl of the lysate was transferred to a GeneJET^{TM} RNA Purification Column inserted in a collection tube and centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was discarded and the column was placed into a new 2 ml RNase-free micro centrifuge tube. 700µl of Wash Buffer 1(supplemented with 250µl of ethanol for every 1ml Wash buffer I) was added to the column and centrifuged for 1 min at 12000 rpm. The flow-through was discarded and 600µl of Wash Buffer II (supplemented with 850µl of ethanol for every 0.5µl Wash buffer II) was added to the column. It was centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was again discarded. Centrifugation was again done at 12000 rpm for 1 min at 4 ° C by adding 250µl of Wash buffer II. The flow-through was discarded and the column was transferred to a sterile 1.5ml RNase-free microcentrifuge tube. 100µl of nuclease-free water was added to the column and centrifuged for 1 min at 12000 rpm to elute RNA. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

RNA quantification by Nanodrop:-

By measuring the absorbance at 260 nm the concentration of the extracted total RNA from cancer tissue was quantified (Table 1) in a nanodrop and calculated by using the formula as given below:

$$\text{Total RNA (µg /ml)} = OD_{260} \times 40 \times \text{Dilution factor.}$$

First strand cDNA synthesis:-

Total RNA (4µg) from cancer tissue were used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler . The RNA were incubated with 1 µl of oligo (dT) primers (100µM, 0.2 µg/µl)

and 12 µl of nuclease-free water at 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spin down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 hr at 42°C. Heating at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0}C for further use.

Gene-specific PCR for amplification of the desired gene:-

Selection of Primers:-

A set of specific forward and reverse primers for the amplification of the desired gene DNMT 3A and DNMT 3B was selected from published papers (Patra, S. K. et al., 2003; Zou et al., 2002). The cDNA of both the blood and cancer tissue synthesized were used as the template for the specific primers. The constitutively expressed housekeeping gene β-actin was used as a positive control to ensure high quality of cDNA. The primer sequences used for the PCR reaction are shown.

PCR amplification of DNMT 3A, 3B gene

For amplification of specific gene sequence such as TLR 22 gene present in cDNA is we required that gene specific forward and reverse primer.

PCR Mixture:- (Total 25µl)

- 0.2 µMdNTP- 0.5µl
- 1.5 mM MgCl2- 1.5µl
- 1x PCR Buffer- 2.5µl
- TaqPolymearse (5U/µl)- 0.5µl
- Primers (0.2µM)- 0.5µl & 0.5µl
- cDNA- 2µl
- MQ Water- 17µl

Methods:-

Autoclaved PCR tubes were labelled according to the samples. A master mix of 1 reaction was prepared in a sterilize eppendrof tube by adding 40.8µl of autoclaved Millipore water, 5µl of 10xTaq assay buffer,1 µl forward primer,1 µl reverse primer, 1 µl dNTPs and 0.2 µl of TaqDNA polymerase, then mixed properly by short spin and kept it in ice.1 µl cDNA was taken from each sample and was put into the labelled PCR tube accordingly, Keep one negative control (without cDNA).The tubes were tapped gently and spined for few seconds. After this the tubes were placed in thermal cycler with program set as follows for DNMT 3A and DNMT 3B:-

94^{c}c for 1 min (Initial denaturation)
94^{0}c for 20 sec (Denaturation)
65^{0}c for 20 sec (Annealing) } 42 cycle
72^{0c} for 30 sec (Extension)
72^{0}c for 5 min (Final extension)

DNMT 3A

94^{c}c for 1 min (Initial denaturation)
94^{0}c for 20 sec (Denaturation)
58^{0}c for 20 sec (Annealing) } 42 cycle
72^{0c} for 30 sec (Extension)
72^{0}c for 5 min (Final extension)

DNMT 3B

4^{0}c hold on forever

- The reaction product was then stored at -20^{0}c until further analysis.
- Then analyse of the PCR amplified product was done in 2% gel.(Fig:- 9)

Gel elution

The DNA fragment of Interest was excise from the agarose gel with a clean, sharp razor blade. Cut away excess gel to minimize the amount of agarose. Then the sliced gel was kept in clean eppendrof tube. 300mL of Gel Solubilization Solution was added to the gel silica. Then the gel mixture was incubated at 50-60 °C for 10 minutes, or until the gel slice is completely dissolved. Vortex briefly every 2-3 minutes during incubation to help dissolve the gel. GenElute Binding Column G was placed into one of the provided 2 ml collection tubes.

Then 500mL of the column Preparation Solution was added to each binding column. Centrifuged for 1 minute and discarded the flow through liquid. Here yellow colour solution was found. 2 gel volumes of 100% isopropanol were added to the solution. Solubilised gel solution mixture was added into the binding column that was assembled in a 2 ml collection tube. Then the column was Centrifuge for 1 minute after loading the column each time. Flow through liquid was discarded. 700mL of Wash Solution was added to the binding column and centrifuged it for 1 minute. The binding column was then removed from the collection tube and discards the flow-through liquid. The binding column was placed back into the collection tube and centrifuge again for 1 minute without any additional wash solution to remove excess ethanol. The binding column was transferred to a fresh collection tube. 50mL of pre heated elution Solution at 65 °C was added to the centre of the membrane and incubated for 1 minute. Then Centrifuge for 1 minute. Supernatant containing DNA was carefully transferred to a new reaction tube. (Fig:-10)

Preparation of ligation mixture

Requirements:-

- 2x rapid ligation buffer (Promega)
- PCR product(Insert)
- T_4 DNA Ligase (Promega)
- Vector-Here I use pBSK(-)(Fig:- 11)

Methods:-

Before use ligation buffer was vertexed properly and then short spinned.

5µl of ligation mixture was prepared in a sterilize eppendrof tube by adding 2.5 µl of ligation buffer,1.5µl of PCR product(Insert),0.5 µl of T_4 DNA ligase and 0.5 µl of pBSK(-) vector. The reaction mixture was mixed properly by pipetting and incubated over night at 4^0c temperature.

Transformation:-

Competent cell preparation:-

For the formation of competent cell we can't take DH5α stain of E.coli directly which is present in glycerol stock solution because here the cell is present in stationary phage. So for this:-

First 200 µl of DH5α stain of E.coli was taken in a eppendroff tube containing 2ml LB. The culture was then incubated over night at 37^0c in water bath shaker to bring the bacteria to their log phase. From this culture 300 µl of DH5α stain was taken in an eppendroff tube containing 100 ml of LB media. Then the tubes were incubated at 37^0 c temp. at water bath shaker .When growth was observed after 2 hr then 12ml of culture was taken in 15ml of tarson tube and immediately place it into ice box. Then the tubes were centrifuged at 5000 RPM for 5 min at 4^0c temperature, supernatant was discarded. The pellets were dissolved into 500µl of 0.1 µl $Cacl_2$.This solution were then transferred into new eppendroff tubes and incubated in ice. After that the tubes were centrifuged at 5000RPM for 5 min at 4^0c and supernatant discarded. The pellets were dissolved into 100 µl of 0.1M $Cacl_2$ and incubated for 15 min into ice.

Incertion of vector to competent cell:-

For the insertion of vector to competent cell exactly 100 µl of competent cell and 5 µl of ligation mixture were taken in a sterilised ependroff tubes. Then the tubes were immediately incubated in ice for 1hr.A brief heat shock was given at 42^0c exactly for 90 sec. and immediately chilling in ice for 15 minutes. Then the contents were transferred into tarson tube containing 1.5ml-2ml LB media. After that the contents were incubated over night at 37^0c at water bath shaker.

Blue white colony selection

The blue-white screen is a molecular technique that allows for the determination of successful ligations in vector-based gene cloning. Insert is ligated into a vector. The vector with insert is then transformed into competent cell(Bacteria). The bacterial cells are then grown in the presence of X-gal. If the ligation process was successful the bacterial colony

will be white; if not, the colony will be blue in colour. This process allows for the quick and easy detection of successful ligation.

The molecular mechanism for blue-white screening is based on a genetic engineering of the *lac* operon. The vector pBlueScript encodes the α subunit of LacZ protein with an internal multiple cloning site (MCS), while the chromosome of the host strain encodes the remaining Ω subunit to form a functional β-galactosidase enzyme. The Multiple cloning site can be cleaved by different restriction enzymes so that the foreign DNA can be inserted within the *lacZ*α gene, because of that disrupting the production of functional β-galactosidase. The chemical required for this screen process is X-gal, a colourless modified galactose sugar that is metabolized by β-galactosidase to form 5-bromo-4-chloro-indoxyl which is spontaneously oxidized to the bright blue in dissolvable pigment 5, 5'-dibromo-4,4'-dichloro-indigo, and because of that functions as a control indicator. Isopropyl β-D-1-thiogalactopyranoside (IPTG), which works as the activator of the Lac operon. This IPTG can be used to enhance the phenotype. The hydrolysis of colourless X-gal by the β-galactosidase causes the characteristic blue colour in the colonies; it shows that the colonies carry vector without insert DNA. White coloured colonies indicate insertion of foreign DNA and loss of the cells ability to hydrolyse the marker.

Colony pick up:-

For this process first LB Agar plate was prepared and kept it for solidification in side tha laminar air flow. After that 50µl of amphicilin was added when temperature falls to 55-60^{0}c then spread this amphicilin with the help of spreader till dry. On that dried LB agar Amphicilin plate 120µl of master mix were added made up of

IPTG-100µl

X-gal-20 µl

Then the master mix was spread with the help of spreader and roter.

After ½ -1hr 10µl of culture were put into the plate and spread well. Before the addition of culture 1st the culture was centrifuged at 5000 RPM for 5 min then thu supernatant were discarded and retained 200µl of supernatant. After that pellet was mixed with the supernatant and spread on the plate. After that the plate was put in side the incubator at 37^{0}c for overnight. Here we found blue-white colonies.(Fig:- 12)

White colonies shoes that these are positive clones mean these bacteria have cloned plasmid. After that the white colonies were picked up with the help of tip and put in the 2ml

eppendroff tube containing 1.5ml lb agar media. Then the tubes were put in overnight incubation at 37^0c temperature at water bath shaker. After maximum growth observed we can proceed for plasmid isolation.

Plasmid isolation from recombinant clones:-

Requirements:-

Alkaline lysis solution I

Alkaline lysis solution II

Alkaline lysis solution III

Antibiotic for plasmid selection

Ethanol

Phenol: Chloroform

LB Broth

Methods:-

White colonies which are expected to be recombinant were picked up with the help of tip and were grown in 1 ml LB media for overnight at 37^0c in water bath shaker. 1.5 ml culture was then transferred into a microcentifuge tube and centrifuge at maximum speed for 30 sec at 4^0C. After centrifugation the supernatant was removed by aspiration, then the pellet was dried as much as possible. The dried bacterial pellet was resuspended by 100µl of ice-cold alkaline solution I by vigorous vortexing. Then 200 µl of freshly prepared alkaline solution II was added to the tube and mixed by inverting the tube rapidly 5 times. Tube was stored on ice. 150 µl of ice-cold alkaline solution III was added. Then the tube was closed and disperse the alkaline lysis solution III through the viscous bacterial lysat by inverting the tubes several times then stored it on ice for 3-5 min. Bacterial lysat was centrifuged at maximum for 5 min at 4^0C and then transferred the supernatant to a fresh tube. 2 volume of ethanol was added to the supernatant and mix it by vortexing and then allow the mixture to incubate for 2 min at room temperature. Then precipitated nucleic acid was collected by centrifugation at maximum speed for 5 min at 4^0C. The supernatant was removed by gentle aspiration and stand the tube in inverted position to allow all the fluid to drain out. 1ml 70%

ethanol was added to the pellet and inverted the tube several time.DNA was recovered by by centrifugation at maximum speed for 2min at 4^0C. Again all supernatant was removed by aspiration. Then the tube was kept in open condition until the ethanol has evaporated. Then the nuclic acid was dissolved in 50 µl of TE containing 20 µg/ml DNase free RNase A. Then plasmid was stored at -20^0C. (Fig: - 13)

Clone Checking:-

After the isolation of Plasmid clone checking was done with the help of 2% gel. Here the vector, Insert (DNMT 3A and DNMT 3B), and isolated plasmid (DNMT 3A and DNMT 3B) were loaded in different lane of the gel(Fig-14).

6.Result

Quantification of RNA by Nanodrop:-

- RNA was isolated from cancer tissue. .
- Quantification was carried out in Nano drop(Table-1).
- The result show an average od value of ~1.6-1.7,conferming the purity of RNA sample.
-

Sample	260/280	Concentration
Sample mix	1.278	116.2 µg/ml

Table .1:-Quantification of RNA

Primer Sequence:-

DNMT 3A:-

Sense :- 5'CACACAGAAGCATATCCAGGAGTG 3'
Anti sense :- 5'AGTGGACTGGGAAACCAAATACCC 3'

DNMT 3B:-

Sence :- 5'AATGTGAATCCAGCCAGCCAGGAAAGGC 3'
Anti sense:- 5'ACTGGATTACACTCCAGGAACCGT3'

PCR using β actin primer for the verification of cDNA:-

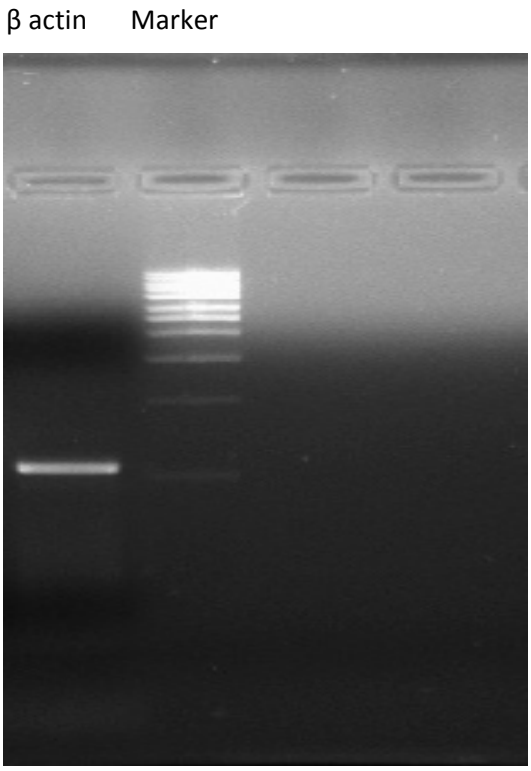
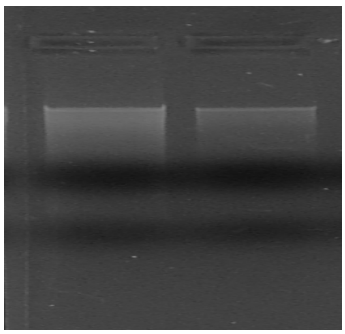
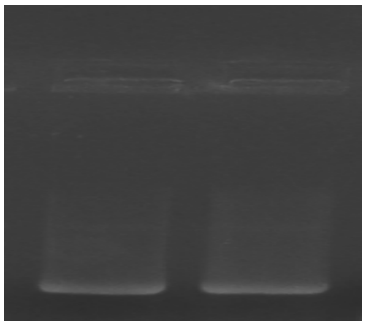


Fig :-7 cDNA checking with β-actin primer

Primer Checking:-



DNMT 3A



DNMT 3B

Fig. 8 Primer Checking

PCR amplification of target genes by desired primers:-

When we amplified prepared cDNA with DNMT 3A and 3B gene specific primer then the primer is amplified only that sequence of cDNA. After amplification we can check it by agarose gel electrophoresis in 1% gel. After complete separation we check it in gel documentation system.

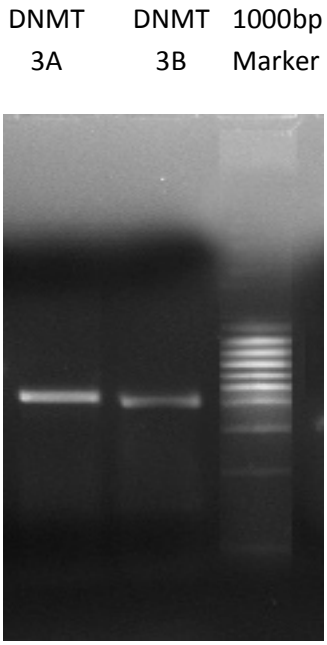


Fig:-9 PCR Amplification

Gel elution of PCR amplified DNA product:-

Before gel illutionAfter gel illution After gel illution

Fig:- 10 Gel elution

Cloning of PCR amplified DNMT 3A and DNMT 3B gene:-

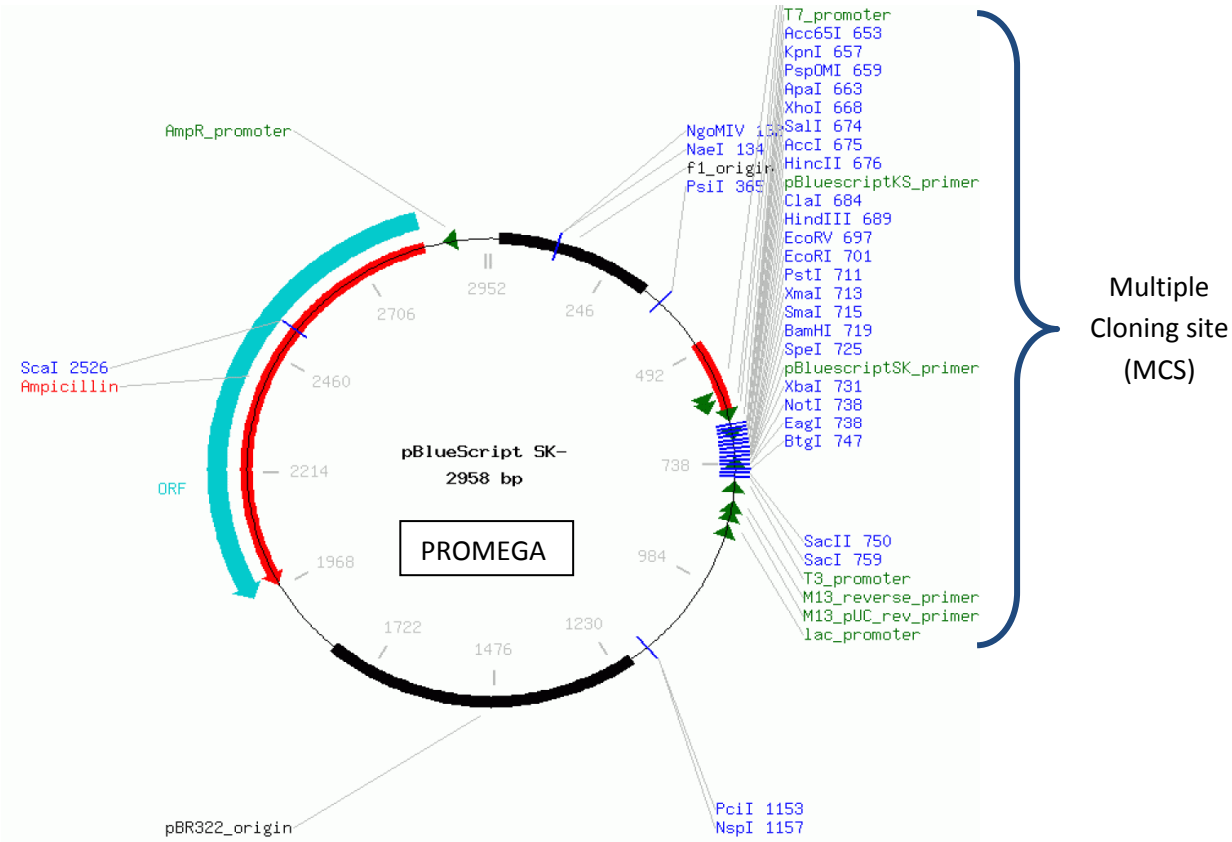


Fig:- 11 pBlueScript SK- vector

To calculate appropriate PCR product amount (insert) the equation is

$$\frac{\text{ng of vector} \times \text{kb size of insert} \times \text{insert : vector molar ratio} = 3:1}{\text{kilo base pair size of vector}}$$

Blue-white colony selection:-

Transformation of competent cell (DH5α) with the ligation mix followed by plating on LB/Amphicillin/IPTG/X-gal plates gives recombinant clones(white colonies) & control clones (Blue colonies) (fig:-7).

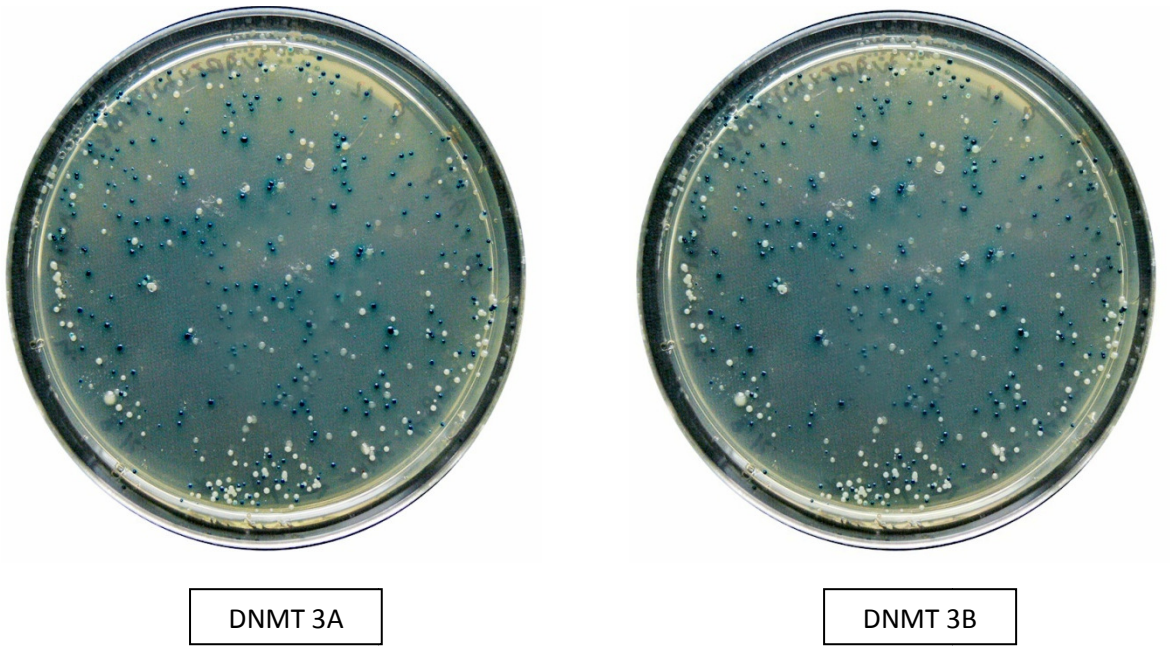


Fig.12:- Blue-white colony selection

Plasmid Isolation:-

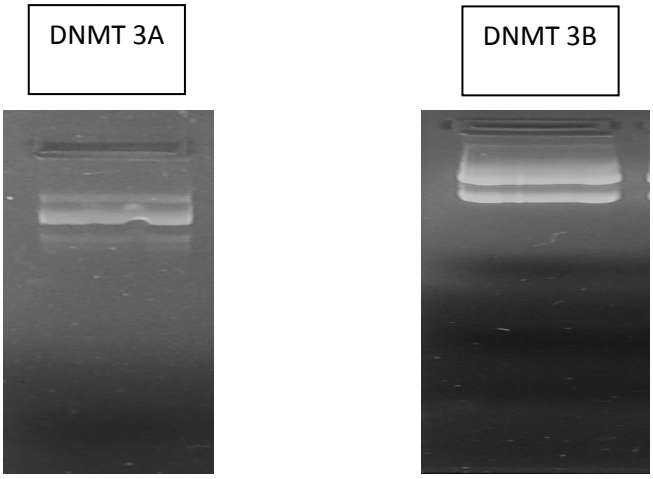


Fig.13:- Plasmid

Clone Checking :-

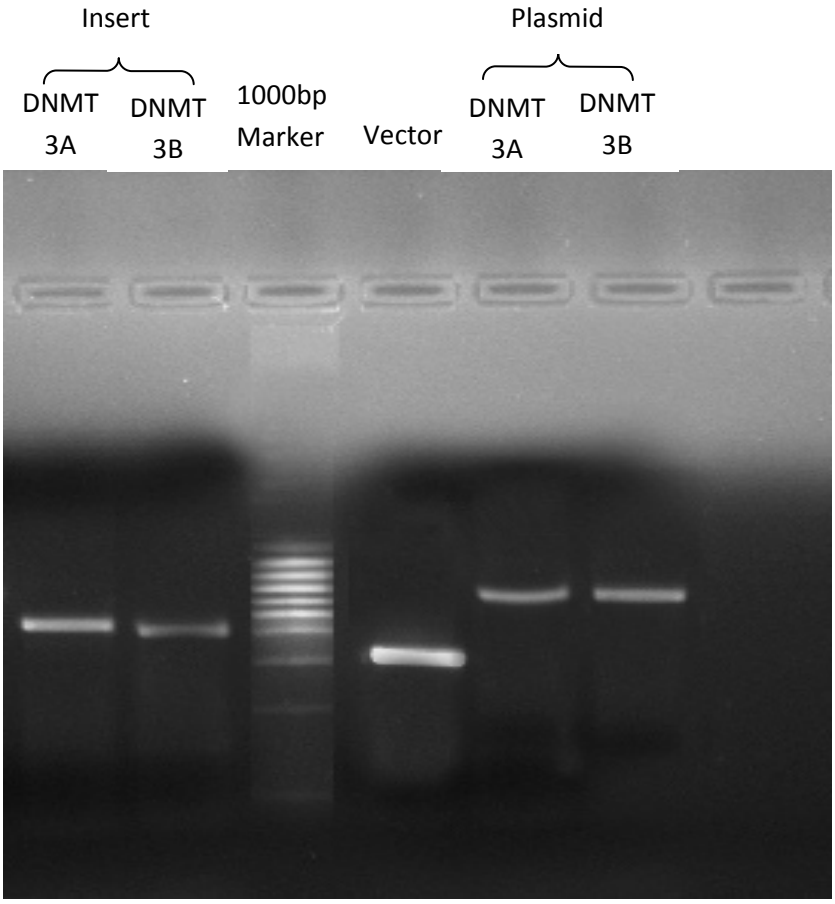


Fig:- 14 Clone checking

7. Discussion

DNA (cytosine-5)-methyltransferase 3A is an enzyme that in humans is encoded by the *DNMT3A* gene And DNA (cytosine-5-)-methyltransferase 3B, also known as DNMT3B, is code for DNMT3B. CpG methylation is an epigenetic modification that is important for embryonic development, genomic imprinting and X-chromosome inactivation. A threshold level of DNA methylation may be required for some essential developmental processes. This optimum methylation level is disrupted during tumorigenesis. Generally DNMT3A and DNMT3B overexpress and help in denovo methylation in cancer cell. In leukemia blood semple DNMT3A and DNMT3B overexpression is related to leukemia development and bad prognosis. DNMT3A and DNMT3B mediated methylation is related to cancer cell proliferation and tumor suppressor genes (TSGs) down regulation in leukemia.

8. Summary and Conclusion

DNMT 3A and DNMT 3B gene were cloned from human cancer tissue which is a highly preferred for their denovo methylation. The de novo activity of DNMT3 and its expression in early development indicate that it is the major enzyme responsible for re-establishing methylation patterns. However, the DNMT3 enzymes are ineffective methylators of the DNA on their own. DNMT3A and DNMT3B cloning increase the chance of cancer research experiment and drug devlopment. Mechanism behind DNMT3A and DNMT3B involvement in cancer development will be clearer after developing DNMT3A and DNMT3B expressing cell. The cloning of the human *DNMT3* genes will facilitate further biochemical and genetic studies of their functions in establishment of DNA methylation patterns, balancing of gene expression and tumorigenesis.

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10. Appendix

1. DEPC treated water

DEPC-Diethyl pyrocarbonate ($C_6H_{12}O_5$). This water is used in laboratory to inactivate the RNase enzyme from water and other lap utensils during RNA extraction which degrade the RNA.It inactivate the RNase by the covelent modification of histidine residue.

For the preparation of DEPC treated water we add 100µl of fresh DEPC to 100 ml oh distil water to make final concentration of 0.5 %(V/V). Then incubated at 37^0C for at least 12hr and then autoclaved at 15lb pressure for 20 min to inactivate the remaining DEPC.

2. Amphicilin stock solution

For the preparation of Amphicilin stock solution amphicilin 1gm was dissolved in 10ml of deionised water and was sterilized by 0.2µm syringe filter. Aliquots were made by distributing 1ml of solution in 1.5 ml appendrof tubes and store at -20^0C.

For LB agar plate concertration is 100µg. For each plate we use 200µl of amphicilin.

3. IPTG stock solution

IPTG-Isopropyl βD thayogalactosidi. IPTG is the inducer of β galactosidase. For the preparation of IPTG stock solution 0.24gm af IPTG was dissolved in 10ml of distilled water then filter sterilised it and stored at -20^0C.

4. X-gal

X-gal:-5-Bromo,4-chloro,3-indolyl,β D galactopyranoside. It is a light sensitive compound. In gene cloning X-gal is used to indicate whether a cell express the β-galactoside enzyme which is encoded by Lac-Z gene in Blue-White screening. When X-gal and IPTG is present in a agar medium on a culture plate colonies which have a functional Lac-Z gene can be easily distinguished. Successful transformed bacteria has truncated β-galactosidase gene causing white colonies on the plate. But bacteri transformed by empty vector which don't contain any insert in

lac Z open reading frame are now able to produce the enzyme-galactosidase which can then cleave X-gal present in nutrient agar , resulting in blue colonies.

For the preparation of X-gal stock solution 100mg of

X-gal was dissolved in 2 ml of N,N',Dimethylformamide,mix well. Then aliquots were prepaired and stored at -20^0C for future use.

5. 75% ethanol

Mix 75ml of absolute ethanol with 25ml of sterile water and not autoclaved, Then stored at-20^0C.

6. TE buffer

Component & final Concentration	Amount to added per 100ml
10mM Tris	1ml (p^H-7.8-8.0 at 25^0C)
1mM EDTA	200µl of 0.5M(P^H-8.0)
H_2O	98.8ml

This standard buffer is used to resuspend and store DNA.

7. 5X TBE buffer

Component & Final concentration	Amount to add per 1 lit
445 mMtris base	54g
245 mM borate	27.5 of boric acid
10mM EDTA	20ml of 0.5 M(P^H-8.0)
H_2O	To make 100 lit